REMARKS

Claims 31-33, 36-41, and 44-50 are currently pending in the present application. Claims 44 and 46-47 have been withdrawn from consideration pending rejoinder.

I. Claim Objections

Claims 39 and 41 have been objected to for containing non-elected species. Applicants submit that the claims are in condition for allowance and request rejoinder of the non-elected species.

II. Rejection Under 35 USC § 112

A. Claims 39 and 41 have been rejected under Sec. 112, first paragraph, for failing to comply with the enablement requirement. The Office alleges that the deposit requirements were not met. Applicants respectfully point out that the verification of deposit was submitted with the June 29, 2005 response (see post card attached). Another copy of the ATCC Patent Deposit Verification is submitted for the Examiner's convenience. Further, as Applicants' representative I stated that the deposit of HB-12699 and HB-12700 was made under the terms of the Budapest Treaty on April 29, 1999, and, upon issuance of a patent from this application, all restrictions imposed upon the deposit would be irrevocably removed. Nothing further is necessary to meet the requirements, and having made the necessary statements and supplied the necessary verification, Applicants request that the rejection be withdrawn.

B. Claims 48 and 50 have been rejected as lacking enablement because they encompass antibodies that would bind to the intracellular or transmembrane regions of the G-CSF receptor. Applicants have amended claim 48 to include the phrase " the extracellular domain of " and therefore request that the rejection be withdrawn.

As to the rejection of claims 31-33, 37-38, 40, and 45, Applicants are confused by the Examiner's statements. The amendment made in the June 29, 2005 response (and in the replacement claims submitted on July 20, 2005 and Oct. 20, 2005), Claim 31 was amended to state the "extracellular domain" whereas Claim 34 was cancelled. Therefore, the rejection of these claims is moot and should be withdrawn.

C. Claim 38 has been rejected because the Office alleges that monovalent fragments would not dimerize the receptor. Although Applicants disagree and maintain that these fragments are useful, the claim has been amended to expedite prosecution and request that the rejection be withdrawn.

III. Rejection Under 35 USC 102(b)

A. Claims 31-33, 36-38, 40, 45 and 48-50 have been rejected as anticipated by Cunningham et al. (U.S. Pat. No. 5,506,107). The Office asserts that "Cunningham et al disclose the production of agonist antibodies which are capable of stimulating receptors for various ligands", and that "production of agonists which stimulate the G-CSF receptor is specifically mentioned at column 12, line 56." The Office further argues that the making of agonist antibodies to hGH anticipates the making of agonist antibodies to G-CSF.

Applicants respectfully traverse this rejection. In order for a reference to qualify as anticipatory it must also be enabling. The mere disclosure of the desire to make an agonist antibody to G-CSF is not enabling, and no one prior to the present inventors had made such an antibody. As discussed in a previous response, the assay disclosed by Cunningham was not sufficiently sensitive to detect agonist antibodies to G-CSF as demonstrated by the present inventors in Figure 5. Moreover, it is easy to obtain a neutralizing antibody because one merely blocks binding of the ligand, i.e. the G-CSF protein, to the receptor, whereas an agonist antibody must bind to the receptor in a proper confirmation and trigger the

activation of the receptor in the same manner as the native ligand. The paper previously submitted by Schneider et al. highlight the difficulty in making dimerizing agonist antibodies and pose the question "Why are agonist antibodies to EPO-R so rare?" highlights the difficulties in obtaining such antibodies. (attached is another copy for your convenience). In that paper, they stated all of their monoclonal antibodies specific for the extracellular domain of EPO receptor should have dimerized the receptor, but in fact only one in the 50 isolated did so. We argue that a similar situation is true for G-CSF, and the assay taught by Cunningham would not lead one to isolate a valid agonist antibody because of the number of false positives.

Thus, due to the rarity of agonist antibodies to EPO, the lack of an adequate assay for detecting agonist antibodies to G-CSF, and the lack of any evidence that an agonist antibody to G-CSF was made prior to the present invention, makes this reference inadequate and lacking enablement to anticipate the currently claimed invention.

B. Claims 31-38, 40 and 45 have been rejected as anticipated by Adams et al. (U.S. Pat. No. 6,342,220). The Office asserts that "Adams et al disclose the production of agonist antibodies which are capable of stimulating receptors for various ligands", and that "production of agonists which stimulate the G-CSF receptor is specifically mentioned at column 12, line 56.

Applicants respectfully traverse this rejection. Adams et al. do not disclose making or any examples of G-CSF agonist antibodies. For the same reasons discussed above, this reference does not qualify as prior art because it does not satisfy the enablement requirement of an anticipatory reference.

Therefore, Applicants submit that the rejections under Sec. 102(b) should be withdrawn.

Conclusion

In view of the foregoing amendments and remarks, Applicants submit that the application is currently in condition for allowance and request a Notice of same.

Respectfully Submitted

Dated: April 10, 2006

BY: Cheryl A. Liljestrand
Cheryl A. Liljestrand
Reg. No. 45,275

AMERICAN TYPE CULTURE COLLECTION
10801 University Blvd.
Manassas, VA 20110-2209
Telephone: 703-365-2700
Fax: 703-365-2745

FACSIMILE

Date: May 7, 1999

To: Eric P. Mirabel
Tanox, Inc.
Fax Number: 713 664 8914

From: ATCC Patent Depository

Number of pages: 1
(Including this page)

REFERENCE: **Patent deposit**

Mouse hybridoma cell line 163-93 assigned ATCC HB-12699 and
Mouse hybridoma cell line 174-74-11 assigned ATCC HB-12700.

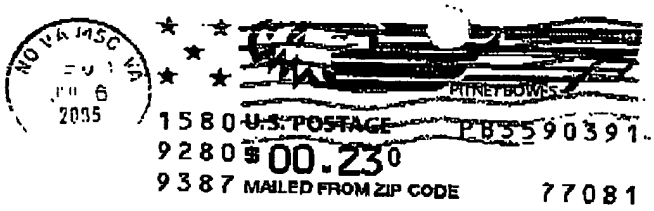
Date of Deposit: April 29, 1999. Paperwork will be forwarded to you in a few days. An invoice will be sent under separate cover:

One time fee - 30 years	$ 1,200.00
Informing fee - 30 years	720.00
Viability Test	200.00
Total amount to ATCC HB-12699 & HB-12700	$ 2,120.00
Balance due to ATCC HB-12699 & HB-12700	$ 2,120.00

Barbara Hailey

Barbara M. Hailey, Administrator
ATCC Patent Depository

Regards



Angela Haffelder
Tanox, Inc.
10301 Stella Link Road
Houston, TX 77025

Please Stamp to Acknowledge Receipt of the Following:

Application of: Baofu NI, et al.
Serial No: 10/071,962
Filed: February 8, 2002
For: G-CSF RECEPTOR AGONIST ANTIBODIES AND SCREENING METHODS THEREFOR

1. Transmittal Form (1 page)
2. Amendment and Response (9 pages)
3. ATCC Deposit Receipt (1 page)
4. Reference: Schneider et al. (10 pages)

Attorney Docket – TNX98-03-01
Dated Mailed: June 29, 2005

Homodimerization of Erythropoietin Receptor by a Bivalent Monoclonal Antibody Triggers Cell Proliferation and Differentiation of Erythroid Precursors

By Helmut Schneider, Warak Chaovapong, David J. Matthews, Cyrus Karkaria, Robert T. Cass, Hangjun Zhan, Mark Boyle, Tony Lorenzini, Steve G. Elliott, and Lutz B. Giebel

Erythropoietin (EPO) stimulates proliferation and differentiation of erythroid progenitor cells. Several lines of evidence indicate that the most likely mechanism of EPO receptor (EPO-R) activation by EPO is homodimerization of the receptor on the surface of erythrocyte precursors. Therefore, we argued that it should be possible to raise EPO-R monoclonal antibodies (MoAbs) that would activate the receptor by dimerization and thus mimic EPO action. We have identified such an agonist MoAb (MoAb34) directed against the extracellular EPO binding domain of the EPO-R. This bivalent IgG antibody triggers the proliferation of EPO-dependent cell lines and induces differentiation of erythroid precursors in vitro, in contrast, the monovalent Fab fragment, which cannot dimerize the receptor, is completely inactive. The mechanism of receptor activation by homodimerization implies that at high ligand concentrations the formation of 1:1 receptor/ligand complexes is favored over 2:1 complexes, thereby turning the ligand agonist into an antagonist. Thus, EPO and MoAb34 should self-antagonize at high concentrations in both cell proliferation and differentiation assays. Our data indeed demonstrate that EPO and MoAb34 antagonize ligand-dependent cell proliferation with IC_{50} values of approximately 20 and 2 μmol/L, respectively. Erythroid colony formation (BFUe) is inhibited at MoAb34 concentrations above 1 μmol/L. Furthermore, we analyzed the MoAb34:EPO-R interaction using a mathematic model describing antibody-mediated receptor dimerization. The data for proliferation and differentiation activity were consistent with the receptor dimer formation on the cell surface predicted by the model.

ERYTHROPOIETIN (EPO), a 34-kD glycoprotein hormone, is the major regulator of mammalian erythropoiesis.[1] EPO acts on erythroid progenitor cells by preventing apoptosis,[2,3] stimulating proliferation of erythroid precursor cells, and inducing differentiation into mature erythrocytes. These effects are transduced by the binding of EPO to a specific EPO receptor (EPO-R) on the surface of committed erythroid progenitor cells.[4] Deletion of EPO and EPO-R genes in mice has shown that EPO is crucial for the survival, proliferation, and differentiation of late committed progenitors (colony-forming unit-erythroid [CFU-E]), but not of early progenitors (burst-forming unit-erythroid [BFU-E]).[5] Mice homozygous for a deletion of either EPO or EPO-R genes die during embryogenesis due to failure of erythropoiesis in the fetal liver. The EPO-R is a member of the cytokine receptor type I superfamily, which includes the receptors for interleukin-2 (IL-2) through IL-7, granulocyte-macrophage colony-stimulating factor, granulocyte colony-stimulating factor (G-CSF), growth hormone (GH), prolactin (PRL), thrombopoietin (TPO), leukemia inhibitory factor, and leptin.[6-8]

The receptors for EPO, GH, PRL, TPO, and G-CSF appear to be triggered by ligand-induced receptor homodimerization.[4,7,9] For the EPO-R, direct evidence for the dimerization model has been provided by the recent discovery of a dimeric peptide that binds to and activates the receptor.[10] The crystal structure of the peptide in complex with the extracellular domain of the EPO-R (EPO binding protein [EPObp]) shows that the peptide dimer binds to two molecules of EPObp.[10] The formation of complexes between EPO and two molecules of EPObp in solution has been described using light-scattering, sedimentation equilibrium, and titration calorimetry techniques.[11] Stable EPObp-EPO complexes have also been purified (Zhan H, Karkaria C, Koc G, Suvel L, Giebel LB, manuscript submitted).

Previous evidence for EPO-induced receptor dimerization on the cell surface is based primarily on constitutively active EPO-R mutants, which contain point mutations introducing cysteine substitutions into the extracellular domain at amino acid positions R129, E132, and E133.[12-14] The EPO-R mutants form disulfide-linked homodimers in the endoplasmic reticulum and on the cell surface.[12] Based on sequence alignments with the related GH receptor, these mutations are expected to be in the receptor-dimer interface region. Expression of the constitutively active EPO-R (R129C) mutant in BaF3 cells results in factor-independent proliferation, and expression in primary cultures of mouse fetal liver cells induces EPO-independent erythroid differentiation.[12] Furthermore, mice infected with a retrovirus carrying the EPO-R (R129C) mutant develop erythroleukemia.[15] Truncated receptor mutants that lack part of the intracellular signaling domain are dominant-negative for signal transduction when coexpressed with the wild-type EPO-R.[16,17] Both wild-type and truncated receptors can be coimmunoprecipitated with an antibody directed against the C-terminus of the wild-type receptor, which is not present in the truncated form,[16] further suggesting the presence of receptor dimers on the cell surface.

Receptor dimerization has been analyzed in great detail for the GH receptor.[18-21] GH has two distinct receptor binding sites. At high ligand concentrations, formation of 1:1 complexes via the high-affinity GH site 1 is favored over 2:1 complexes, preventing GH receptor signaling, and Puh et

From the Department of Receptor Biology, Arris Pharmaceutical Corp, South San Francisco; and the Amgen Center, Amgen, Thousand Oaks, CA.

Submitted May 1, 1996; accepted September 3, 1996.

Address reprint requests to Helmut Schneider, PhD, Arris Pharmaceutical Corp, 385 Oyster Point Blvd, South San Francisco, CA 94080.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. section 1734 solely to indicate this fact.

0006-4971/97/8902-0026$3.00/0

al[23] demonstrated self-antagonism of GH in a GH-dependent cell proliferation assay at GH concentrations greater than 100 nmol/L. Homodimerization and signal transduction of GH receptor can also be achieved by specific monoclonal receptor antibodies. IgG-type antibodies are bivalent molecules that bind to two antigen molecules at the same time. IgGs specific to GH receptor therefore are able to dimerize and stimulate the receptor. Like GH, these antibodies are self-antagonists at high concentrations.[23] Similar agonist antibodies have also been described for the PRL receptor.[25]

We have raised a monoclonal antibody (MoAb) directed against EPObp, which mimics EPO action by inducing ligand-dependent cell proliferation and differentiation. Self-antagonism of EPO has not been reported so far, probably because the concentrations of EPO tested have not been high enough. We analyzed EPO at concentrations up to 30 μmol/L and were able to detect specific inhibition of EPO-dependent proliferation. This provides further evidence that EPO triggers its receptor by ligand-induced dimerization.

MATERIALS AND METHODS

Cell lines. BaF3, a murine IL-3–dependent cell line,[24] was a gift from Dr H. Ludish (Whitehead Institute, Cambridge, MA). An EPO-dependent BaF3/EPO-R cell line was generated by transfecting the full-length human EPO-R into BaF3 cells. A cDNA encoding the full-length human EPO-R (a gift from Dr J. Winkelmann, University of Cincinnati, OH; nucleotide sequence identical to that used by Winkelmann et al[25] and Jones et al[26]) was cloned into plasmid expression vector pRc/CMV (Invitrogen, San Diego, CA). After electroporation into BaF3 cells, the cells were cultured for 2 days in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and IL-3. Cells were washed twice, transferred into RPMI 1640 medium plus 135 pmol/L EPO, and selected for EPO-dependent growth. Individual clones were selected by limiting-dilution cloning. The EPO-dependent cell line chosen for this study proliferates in the presence of EPO with an EC_{50} of 15 pmol/L. Scatchard analysis showed 800 receptors per cell that bind EPO with 300-pmol/L affinity (if, for simplification, single-site binding is assumed (data not shown). Cells were maintained in RPMI 1640 medium with 10% FBS, 20 mmol/L HEPES, pH 7.8, and 10 μmol/L mercaptoethanol supplemented with 100 pmol/L EPO. BaF3 cells were supplemented with 10% IL-3 containing WEHI-3B–conditioned medium.

UT-7/EPO cells[27] were a gift from Dr Norio Komatsu, and were grown in 1X Iscove's modified Dulbecco's medium (IMDM) with L-glutamine, 25 mmol/L HEPES, 3.024 g/L sodium bicarbonate, 10% FBS, and 1% L-glutamine-penicillin-streptomycin solution (Irvine Scientific, Santa Ana, CA) containing 270 pmol/L EPO.

Expression and affinity purification of soluble human EPO-R. DNA encoding a soluble truncated EPO-R (EPObp) was generated by polymerase chain reaction (PCR) using the full-length cDNA as template. The amplification product introduces a TAG termination codon 5′ of the transmembrane region and encodes the extracellular domain consisting of amino acids 1 through 249 of the published sequence.[26] The PCR product was subcloned into expression vector pRc/CMV (Invitrogen) and stably transfected into CHO cells. Individual clones secreting EPObp were selected by limiting-dilution cloning. Roller bottles (surface area, 1,700 cm²; Corning, Corning, NY) were seeded with the stable cell line, which was then grown to confluence in RPMI 1640 medium plus 10% FBS. The cells were washed twice in serum-free RPMI 1640 medium and cultured in 200 mL serum-free RPMI 1640 medium. Cell supernatant was collected after 2 days, and fresh medium was added for another 2 days.

EPO was oxidized with 10 mmol/L $NaIO_4$ and biotinylated using 10 mmol/L biotin hydrazide (Pierce, Rockford, IL) following the manufacturer's instructions. A ligand affinity column was prepared by immobilizing biotinylated EPO (10 mg) on Streptavidin 3M Emphaze beads (3 mL; Pierce) overnight in Dulbecco's phosphate-buffered saline (PBS) (Irvine Scientific) at 4°C. The beads were separated from the supernatant by centrifugation, and incubated with 10 mmol/L biotin in PBS for 2 hours at 4°C to saturate all biotin binding sites. After washing with PBS, the EPO-coated beads were packed in a glass column (Omnifit; Alltech Corp, Deerfield, IL). Cell supernatant (10 L) was concentrated and diafiltered to 1 L in 20 mmol/L Tris hydrochloride, pH 7.6, and loaded on the column at a flow rate of 0.7 mL/min. The column was washed with 50 mL 20-mmol/L Tris hydrochloride, pH 7.6. Bound EPObp was eluted with 750 mmol/L NaCl in 20 mmol/L Tris hydrochloride, pH 7.6. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis showed a single 30-kD EPObp band. The EPObp fractions were pooled and concentrated, and the buffer was exchanged with PBS to a final concentration of 0.8 mg/mL.

Generation and screening of MoAb. Five balbic mice were immunized by seven subcutaneous injections at two sites over a period of 14 weeks. Each 50-μL injection contained 25 μg EPObp in Freund's adjuvant. After 12 weeks, all mice had developed anti-EPObp antibody titers. The dilutions needed to reach a half-maximal signal in an enzyme-linked immunosorbent assay (ELISA) ranging from 1:20,000 to 1:50,000. An additional final injection of 150 μg EPObp was administered intravenously to the mouse expressing the highest antibody titer. After 3 days, spleen cells were isolated and fused with myeloma cells P3X63Ag8.653 (American Type Culture Collection, Rockville, MD: CRL 1580). After selection in hypoxanthine-aminopterin-thymidine medium (Sigma, St Louis, MO) for 10 days, a total of 475 supernatants were screened for specific antibody production by ELISA. Positive clones were transferred to 24-well microtiter plates, and supernatants were assayed in a thymidine uptake proliferation assay using the cell line BaF3/EPO-R and the parental BaF3 as a control. Hybridoma clone MoAb34 was subcloned twice by limiting dilution. Ig isotyping was performed using the IsoStrip Mouse Monoclonal Antibody Isotyping Kit from Boehringer Mannheim (Indianapolis, IN).

Purification of MoAb34 and Fab preparation. Hybridomas were grown in 47.5% RPMI 1640 medium, 47.5% Dulbecco's modified Eagle's medium, and 5% FBS. Culture supernatant was filtered through a 0.2-μm membrane. A 6-mL protein G–Sepharose 4 fast-flow column (Pharmacia Biotech, Piscataway, NJ) was packed with 80 psi pressure. A 1-L sample was loaded at 4 mL/min at 4°C, followed by washing with greater than 5 column vol PBS. MoAb34 was then eluted from the column with ImmunoPure IgG elution buffer (Pierce) at 2 mL/min. The eluate was immediately neutralized to pH 7.5 by adding 3 mol/L Tris. The purity was evaluated by nonreducing SDS-PAGE. Fab fragments were generated by papain cleavage using the ImmunoPure IgG1 Fab Preparation Kit (Pierce) following the manufacturer's instructions. The Fab was further purified by gel filtration using a Superdex 75 column (1.6 cm × 60 cm; Pharmacia), eluted with PBS, and analyzed for purity by SDS-PAGE.

ELISA. Because we expected the number of agonist antibodies to be low, we used two different methods for immobilization of the EPObp to ensure identification of a maximum number of anti-EPObp MoAbs. In ELISA 1, EPObp was covalently immobilized. EPObp was oxidized in 1 mmol/L $NaIO_4$ and 50 mmol/L sodium acetate, pH 5.5, at 4°C for 30 minutes in the dark. The protein was separated from periodate on a NAP-5 column (Pharmacia) and incubated on hydrazide-activated microtiter plates (Unisyn, San Diego, CA) at 2 μg/mL (100 μL per well) for 1 hour at room temperature. Plates were washed and blocked with PBS and 20 mg/mL bovine serum albumin (BSA) for 1 hour. In ELISA 2, Polysorb microtiter plates

(Nunc, Roskilde, Denmark) were incubated for 1 hour at 37°C with 10 μg/mL MoAb 2E12, a specific, nonneutralizing rat MoAb directed against EPObp (B. van Dyke, personal communication, February 1994). The plates were washed and blocked, and then EPObp (1 μg/mL) was added in ELISA buffer (PBS, 1 mg/mL BSA, and 0.02% Tween-20) for 1 hour at 37°C. After immobilization of EPObp, both ELISA protocols were identical. Antibody-containing samples in ELISA buffer were added and incubated for 1 hour at 37°C. After washing, plates were incubated with a sheep anti-mouse IgG coupled to horseradish peroxidase (HRP; Sigma) at 0.1 ng/mL in ELISA buffer for 1 hour at 37°C. The plates were then washed, and 100 μL TMB/H_2O_2 developing solution (Pierce) was added and incubated for 5 minutes. Color development was stopped by adding 100 μL 1-mol/L sulfuric acid, and the $OD_{450-650}$ was determined using a plate reader (Molecular Devices, Sunnyvale, CA).

Thymidine uptake proliferation assays. BaF3 and BaF3/EPO-R cells were grown to the late logarithmic phase, collected by centrifugation, washed three times with RPMI 1640 media containing 10% FBS and 10 mmol/L HEPES, pH 7, in the absence of EPO and IL-3, and then starved in the same media for 2 hours. Antibody test samples (hybridoma supernatants or purified proteins) were diluted at least fourfold into 100 μL media, and 100 μL cells were added (25,000 cells per well). EPO was dialyzed against 10 mmol/L HEPES, pH 7.0, and 100-μL test samples were combined with 100 μL cells (25,000 cells per well) in twofold-concentrated medium. Plates were incubated for 4 hours at 37°C and 5% CO_2 in a humidified tissue culture incubator. Then, 0.5 μCi methyl-[^{3}H]thymidine (Amersham, Arlington Heights, IL; 1 mCi/mL, 20 Ci/mmol) diluted into 20 μL medium, was added, and the incubation was continued for another 15 hours. Cells were harvested onto glass fiber filtermats using a Tomtec cell harvester, and incorporated radioiabel was determined using a Microbeta 1450 scintillation counter (Wallac, Turku, Finland).

UT-7/EPO cells were grown to approximately 3×10^5/mL, collected by centrifugation, washed twice with PBS, and resuspended at 50,000/mL in assay medium (RPMI 1640 medium with 1% L-glutamine and 4% FBS). Tissue culture plates (96-well) were loaded with 100-μL test samples (diluted at least fivefold in assay medium) and 50 μL cells per well and incubated at 37°C and 5% CO_2. After 72 hours, 0.5 μCi methyl-[^{3}H]thymidine diluted in 50 μL assay medium was added, and the cells were incubated for another 4 hours at 37°C and 5% CO_2. Labeled cells were harvested onto glass fiber filtermats using a PHD cell harvester (Cambridge Technology, Inc, Watertown, MA). Filters were rinsed with 2-propanol, dried, and counted in a Beckman model LS6000IC scintillation counter (Fullerton, CA).

Cell-based EPO binding-competition assay. OCIM1 cells, a human erythroleukemia cell line that expresses EPO-R on the cell surface,[29] were grown in IMDM, 10% FBS, and 1% penicillin-streptomycin to approximately 2 to 5×10^5 cells/mL. Cells were collected by centrifugation, washed two times in binding buffer (RPMI 1640 medium, 1% BSA, and 25 mmol/L HEPES, pH 7.3), and resuspended in binding buffer containing 0.1% NaN_3 and 10 μg/mL cytochalasin B at 1 to 2×10^7 cells/mL. Tissue culture plates (96-well) were loaded with 100 μL cells, 10 μL sample, and 50 μL [^{125}I]-EPO (Amersham, high specific activity, 3,000 Ci/mmol, 2 mCi/mL) and incubated for 3 hours at 37°C in a humidified tissue culture incubator. Then, the cells were centrifuged through phthalate oil (6b:4f) (a/v/v) dibutylidimonyl phthalate) in filter tubes. The tubes containing cell pellets were quick-frozen in a dry ice–ethanol bath, and the cell pellet was clipped and then counted in a Pharmacia-LKB (Uppsala, Sweden) 1277 Gammamaster automatic gamma counter.

BFU-e cell differentiation assay. Normal human donors underwent lymphapheresis according to standard protocols to purify CD34$^+$ erythroid cells.[29] Human blood was obtained after informed consent. The cells were washed, resuspended in Hanks balanced salt solution (HBSS), and separated by density centrifugation over a gradient (Ficoll-Paque; Pharmacia Biotech). The low-density cells were collected, washed with HBSS, and resuspended in PBS supplemented with 0.5% BSA and 5 mmol/L EDTA at a concentration of 5×10^8 cells/mL. From these cells, purified CD34$^+$ cells were obtained using a CD34 Progenitor Cell Isolation Kit (QBend/10; Miltenyi Biotech GmbH, Bergisch Gladbach, Germany).

The in vitro BFU-E assay on purified CD34$^+$ cells was performed in methylcellulose. The medium contained 20% FBS, 0.33X IMDM (GIBCO, Grand Island, NY), salts, 2-mercaptoethanol, nucleosides, cholesterol, sodium pyruvate, Human transferrin, lipids, Human-insulin, deionized BSA, and 100 ng/mL stem cell factor (SCF).[30] A suspension of CD34$^+$ cells (10,000/mL), 0.015 mL SCF (20 μg/mL), and a combination of sample and medium totaling 3 mL was prepared in sterile polystyrene tubes. Duplicate 1-mL aliquots were plated onto 35 × 100-mm tissue culture plates. The plates were incubated at 37°C and 10% CO_2 in a humidified tissue culture incubator. Erythroid colonies (orange to red in color) were scored after 19 to 20 days.

BIAcore analysis. Kinetic parameters for the interaction of MoAb34 and its Fab fragment (Fab34) with EPObp were measured using real-time biospecific interaction analysis (BIAcore).[31] The BIAcore system, CM-5 sensor chip, and reagents were from Pharmacia Biosensor (Piscataway, NJ). All injections on the sensor chip surface were at a flow rate of 5 mL/min and 25°C unless otherwise stated. Between injections of reagents, the sensor chip was continuously washed with 10 mmol/L HEPES, pH 7.2, 150 mmol/L NaCl, 3.4 mmol/L EDTA, and 0.005% surfactant P_{20}. The interaction of MoAb34 with EPObp was characterized by coupling approximately 6,800 resonance units (RU) of MoAb34 to the sensor chip surface using standard amine immobilization chemistry.[32] EPObp samples of 10 to 1,500 nmol/L were injected for 7 minutes over the MoAb34 surface and over a control flow cell. After each injection of EPObp, a 1-minute pulse of 1 mmol/L formic acid was used to regenerate the MoAb34 surface. To measure the interaction of EPObp and Fab34, oxidized EPObp (~500 RU) was immobilized via carbohydrazide coupling to the carboxymethylated dextran matrix.[33] Injections of Fab34 spanned a concentration range of 1 to 500 nmol/L. After each injection of Fab34, the EPObp surface was regenerated with a 50-μL pulse, at 50 μL/min, of 10 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS; pH 10.4). Association and dissociation rate constants were determined by least-squares fitting using BIAevaluation software (Pharmacia Biosensor). To minimize potential rebinding effects, only the initial 15 seconds of each dissociation profile was used for calculation of the dissociation rate constant.

RESULTS

MoAb34 stimulates proliferation of BaF3/EPO-R cells. We have isolated a total of 48 MoAbs specific for EPObp,

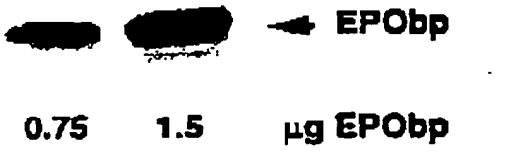


Fig 1. MoAb34 immunoblot analysis of EPObp. EPObp was heat-denatured and analyzed by reducing SDS-PAGE on a 12% acrylamide gel and subsequent transfer to nitrocellulose. The blot was incubated with MoAb34 (10 μg/mL) and subsequently with antimouse IgG coupled to horseradish peroxidase.

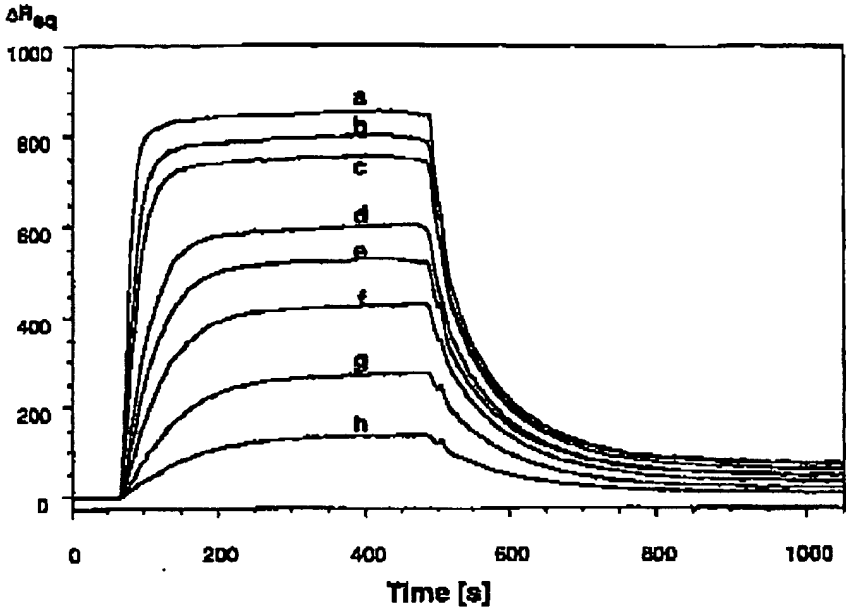


Fig 2. BIAcore analysis of MoAb34. Sensorgrams of various concentrations of EPObp (10 to 1,500 nmol/L) injected over immobilized MoAb34, corrected by subtraction of data from control surfaces. The ligand was removed after 420 seconds, and dissociation was measured after this point. Data from 8 representative free-EPObp concentrations (nmol/L) from a total of 16 are shown: 1,500 (a), 750 (b), 150 (c), 100 (d), 75 (e), 50 (f), 25 (g), and 10 (h).

the soluble extracellular ligand-binding domain of the human EPO-R. The hybridoma supernatant of MoAb clone 34 stimulated thymidine uptake in BaF3/EPO-R cells, but did not stimulate proliferation in parental BaF3 cells. MoAb34 is an IgG MoAb that was subtyped as IgG_{2a}. Immunoblot analysis of heat-denatured and reduced EPObp suggested that MoAb34 recognizes a linear epitope (Fig 1). MoAb34 did not compete with [^{125}I]-EPO in a binding-competition assay using human OCIM1 cells[28] (data not shown). Thus, MoAb34 does not interfere with the binding of EPO to its receptor, indicating that there is no overlap of MoAb34 and EPO binding epitopes on the receptor. The binding kinetics of both MoAb34 and Fab34 to EPObp were characterized using surface plasmon resonance (Fig 2). For MoAb34 kinetics, the antibody was immobilized to avoid avidity effects. For Fab34 kinetics, we immobilized EPObp. The kinetic constants for MoAb34 and Fab34 are summarized in Table 1.

MoAb34, but not the Fab fragments, stimulate proliferation in EPO-dependent cells. Purified MoAb34 was able to stimulate proliferation in EPO-dependent cell lines. A dose-dependent response evaluation in a [^{3}H]thymidine uptake cell proliferation assay showed EC_{50} values of approximately 10 nmol/L (Fig 3A) in BaF3/EPO-R cells. The effect of MoAb34 was specific to EPO-R, because it did not stimulate growth of the parental BaF3 cell line (data not shown). Under identical conditions, the maximal amount of [^{3}H]-thymidine incorporation caused by EPO (Fig 4) was eight-fold to 10-fold higher than the incorporation caused by MoAb34 (Fig 3A). In contrast to the bivalent MoAb34, monovalent Fab fragments did not stimulate proliferation of the BaF3/EPO-R cell line (Fig 3A), even though the affinities of MoAb34 and Fab34 to EPObp were similar (Table 1). MoAb34 was even more active in the cell line UT-7/EPO,[27] which expresses endogenous EPO-R, where it stimulated proliferation with an EC_{50} of approximately 300 pmol/L (Fig 3B). The maximum incorporation was close to the value obtained with EPO. This may be due to the higher concentration of EPO-R molecules on the surface of UT-7/EPO cells, which contain 2,400 receptors per cell,[27,34] versus 800 for BaF3/EPO-R (data not shown). For higher receptor concentrations, the ligand concentration necessary to induce dimerization of the receptors should be lower. At higher concentrations (>200 nmol/L), MoAb34 antagonizes cell proliferation in both cell lines (Fig 3), as expected based on the homodimerization model. The resulting dose-dependent proliferation curves have a bell-shaped character with IC_{50} values for self-antagonism of approximately 2 μmol/L.

If EPO homodimerizes the receptor, then self-antagonism should also be observed at high EPO concentrations. We demonstrated this using our BaF3/EPO-R cell proliferation assay (Fig 4). Proliferation significantly decreased above 3 μmol/L; however, complete inhibition was not observed at the concentrations tested. The estimated IC_{50} value was approximately 20 μmol/L, representing 74,000 U/mL or 370 μg/mL of EPO. This is an extremely high concentration—BaF3/EPO-R cells proliferate with an EC_{50} of 15 pmol/L, which is six orders of magnitude lower. To confirm that the decrease in signal in BaF3/EPO-R cells was specific to EPO and not due to toxicity or other artifacts at such high ligand concentrations, parental BaF3 cells were incubated with EPO at identical concentrations in the presence of IL-3. No decrease in IL-3–dependent

Table 1. BIAcore Analysis of MoAb34 and Fab34 Interactions With EPObp

Immobilized Molecule	Analyte	K_a (mol/L^{-1} s^{-1})	K_d (s^{-1})	K_D (nmol/L)
MoAb34	EPObp	3.1×10^6	0.026	84
EPObp	Fab34	4.9×10^5	0.026	53

Determinations of affinity were made from kinetic measurements of K_a and k_d, and K_D was inferred from the relationship $K_D = k_d/K_a$. Standard errors in all cases were <10%.

proliferation was observed at any EPO concentration (Fig 4).

MoAb34 induces differentiation of CD34$^+$ erythroid progenitor cells in the presence of SCF. The differentiation

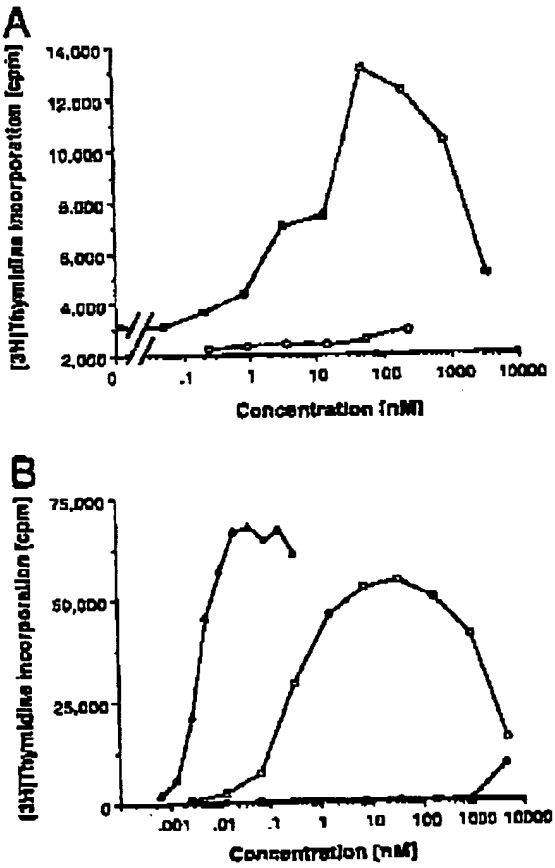


Fig 3. Dose-dependent proliferation of EPO-sensitive cells by MoAb34. (A) Proliferation of BaF3/EPO-R cells in the presence of [^{3}H]-thymidine and various concentrations of MoAb34 (□) and Fab34 (○). (B) Proliferation of UT-7/EPO in the presence of [^{3}H]thymidine and various concentrations of MoAb34 (□), control (anti-Axl) antibody (●), and EPO (Δ), respectively. Experiments were made in duplicate; the mean values are shown.

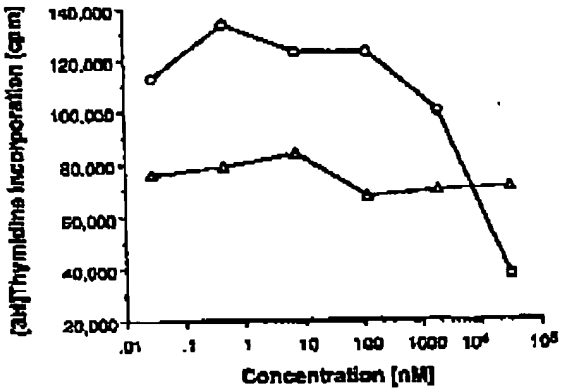


Fig 4. Effect of high EPO concentrations on the proliferation of BaF3/EPO-R cells (○) and parental BaF3 cells (Δ). Both cell lines were assayed with identical dilution series of EPO. BaF3 cells were supplemented with 10% WEHI-conditioned media as a source for IL-3. Experiments were made in duplicate; the mean values are shown.

of BFUe cells in the CD34$^+$ population to erythroid bursts is dependent on EPO and SCF. MoAb34 was able to induce in vitro differentiation of human CD34$^+$ erythroid cell precursors. In two independent experiments, duplicate sets of CD34$^+$ cells were treated with various concentrations of MoAb34, EPO, or a control antibody. The cells were incubated in the presence of a fixed concentration of SCF (100 ng/mL). After 19 days in the first experiment and 20 days in the second experiment, colonies from BFUe cells were visible in the presence of either MoAb34 or EPO, but not in the antibody control (Table 2). The colonies showed typical red color and could be identified as erythroid cells by microscopic analysis (Fig 5). As in the cell proliferation assays described earlier, EPO was more potent than MoAb34. Colonies developed at the lowest EPO concentration tested (1.3 pmol/L), whereas no erythroid colonies were observed at a MoAb34 concentration less than 7 nmol/L. Both the absolute number of colonies and the size of the colonies were higher in the presence of EPO than in the presence of MoAb34. The approximate EC_{50} value was 15 nmol/L, and the highest stimulation of differentiation by MoAb34 observed was 22 to 220 nmol/L, similar to the maxima observed in the cell proliferation assays (Table 2). In addition, at concentrations above 720 nmol/L, MoAb34 self-antagonizes in this cell differentiation assay. All these data demonstrate that both cell proliferation and differentiation are driven by ligand-induced receptor homodimerization.

Agonist activity of MoAb34 correlates well with a model for antibody-mediated receptor dimerization. Mathematic models have been developed to describe the formation of receptor dimers on the cell surface by bivalent ligand antibodies[25] or by GH.[26] We investigated how the agonist activity of MoAb34 would correlate with the occurrence of receptor dimers predicted by the model of Perelson.[25]

Briefly, Perelson postulates a two-step mechanism, whereby the formation of 1:1 complexes is driven by the

affinity constant K_A (= 1 K_D). Subsequent dimer formation is dependent on a "cross-linking" constant K_X, which includes K_A but also depends on the effective concentration of receptors on the cell surface and other factors. In the following equations, "Ab" stands for antireceptor antibody and "R" for receptor:

$$[Ab] + [R] \overset{K_A}{\leftrightarrow} [Ab.R]$$

$$[Ab.R] + [R] \overset{K_X}{\leftrightarrow} [R.Ab.R].$$

The concentration of dimer is calculated as

$$[dimer] = \frac{[R_{total}]}{2}\left\{1 + \frac{1}{3\delta}(1 - \sqrt{1 + 4\delta})\right\},$$

$$\text{where } \delta = \frac{[Ab][R_{total}]K_AK_X}{(1 + 2K_A[Ab])^3},$$

assuming that the amount of antibody bound is small compared with the total antibody concentration. The maximal concentration of dimer is solely dependent on K_A:

$$[dimer]_{max} = \frac{1}{2K_A} = \frac{1}{2}K_d.$$

If the percentage of receptor/antibody 2:1 complexes versus the total number of receptors is plotted against the antibody concentration, a symmetric bell-shaped curve is predicted. The maximum of 2:1 complexes occurs at a defined antibody concentration equal to half the antibody K_D value. Details of the above derivation may be found elsewhere.[32]

Figure 6 fits the data of the MoAb34 cell proliferation and differentiation assays to the equation. The resulting bell-shaped curves for the proliferation assays correlate well with the assay data. The obtained 2:1 complex maxima were 114 nmol/L for BaF3/EPO-R cells and 26 nmol/L for UT-7/EPO cells. According to the model, this translates to apparent K_d values of 228 and 52 nmol/L, respectively. In good agreement with the K_d value of 84 nmol/L determined by BIAcore analysis (Table 1). These results demonstrate that the agonist activity of the bivalent MoAb34 in cell proliferation and differentiation assays is consistent with ligand-induced homodimerization of the EPO-R on the cell surface.

DISCUSSION

Homodimerization of the EPO-R by EPO on the cell surface is believed to be the key event in signal transduction.[4] The model for homodimerization of the EPO-R by EPO implies that it should be possible to trigger the receptor by

Table 2. BFUs in Vitro Differentiation Assay

Factor	Experiment 1 (19 d) Concentration (nmol/L)	Experiment 1 (19 d) No. of Colonies (duplicates)	Experiment 2 (20 d) Concentration (nmol/L)	Experiment 2 (20 d) No. of Colonies (duplicates)
EPO			0.0013	9/11
	0.0026	30/23	0.0026	31/27
	0.0065	31/37	0.0065	48/62
	0.0130	48/56	0.0130	81/87
	0.0259	55/61	0.0259	142/100
	0.0649	73/63		
			0.130	133/128
MoAb34			1.8	5/0
	3.6	0/0	3.6	0/0
	7.2	0/2	7.2	0/0
			14.4	5/6
	21.6	12/10	21.5	10/7
	36.0	15/3	36.0	13/12
	72.0	10/7	72.0	7/15
			144	5/14
	216	11/4	216	4/13
	360	4/3	360	4/3
	720	2/0	720	0/2
	1,080	0/1	1,080	0/0
	1,800	0/1		
MoAb control			6.9	0/0
	34.4	0/0	34.4	0/0
	206	0/0	206	0/0
	344	0/0		
			688	0/0

Purified CD34$^+$ cells (10,000 cells per duplicate sample) were incubated in methylcellulose in the presence of the indicated samples at different concentrations. Erythroid colonies were counted after 19 days (experiment 1) or 20 days (experiment 2), respectively. For each experiment, the number of colonies of both duplicates are given.

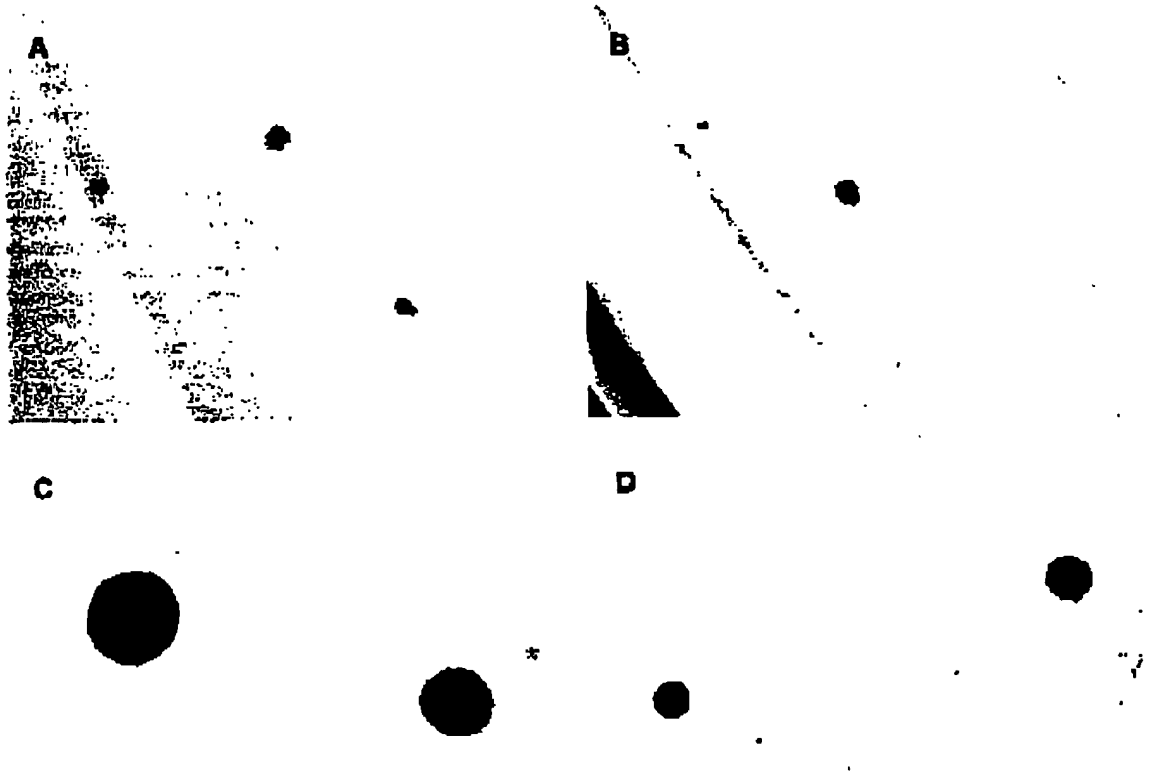


Fig 5. BFUe colonies stimulated by MoAb34 and EPO. Purified CD34+ cells from human peripheral blood were incubated for 20 days. BFUe colonies were identified by microscopic examination and photographed. Representative colonies obtained at 2 different concentrations of EPO and MoAb34, respectively, are shown. (A) 144 nmol/L MoAb34; (B) 36 nmol/L MoAb34; (C) 130 pmol/L EPO; (D) 6.8 pmol/L EPO.

bivalent MoAbs directed against EPObp. For the related GH receptor, a variety of agonist MoAbs have been reported.[23] MoAb34 is such a bivalent IgG with the ability to dimerize two receptor molecules. We have shown stimulation of cell proliferation in BaF3/EPO-R and UT-7/EPO, as well as stimulation of cell differentiation in CD34+. On the other hand, monovalent Fab fragments, which cannot form receptor dimers, are totally unable to stimulate cell proliferation in BaF3/EPO-R, although their affinity for EPObp is similar. In all three test systems used, we observed self-antagonism of MoAb34 at high concentrations. This is precisely what the homodimerization mechanism requires: when high ligand concentrations drive the equilibrium from 2:1 complexes toward 1:1 complexes, there are fewer receptor dimers on the cell surface triggering signal transduction. Self-antagonism has been described for agonist GH receptor and PRL receptor antibodies, too,[23,28] suggesting that all three receptors are activated by the same principle.

Over the full antibody concentration range, activation of cell proliferation and differentiation shows a bell-shaped response curve. The maximum observed in all three in vitro experiments occurs at similar MoAb34 concentrations, in close vicinity to its K_d. Indeed, a mathematic model[35] predicts that a maximum of 2:1 receptor/antibody complexes is formed at a concentration of 0.5 × K_d. We were able to fit the data obtained in the cell proliferation and differentiation assays to this model. The correlation of agonist activity with the predicted occurrence of 2:1 complexes further supports the homodimerization model. In contrast, data reported for agonist antibodies stimulating the GH and PRL receptor show a maximum response in proliferation assays at concentrations approximately two orders of magnitude higher than their K_d values.[23,28] It is possible that the affinities of anti-PRL receptor antibodies were overestimated due to avidity effects, since they were determined by binding analysis of radiolabeled antibodies to whole-cell homogenates.[39] In the GH-GH receptor complex, there is a substantial contact surface between the two GH receptor molecules in addition to

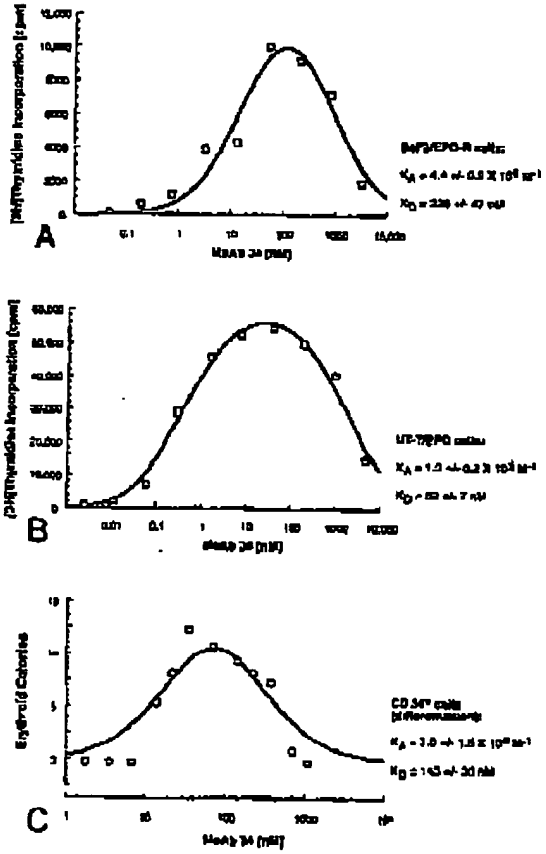


Fig 6. Fit of data obtained from in vitro cell assays to a mathematic model describing antibody-mediated receptor dimerization. Determination of K_D by BIAcore analysis showed a value of ~34 nmol/L. Baseline values for proliferation of [^{3}H]thymidine incorporation in the absence of MoAb34 were subtracted. (A) Proliferation of BaF3/EPO-R cells; (B) proliferation of UT-7/EPO cells; (C) differentiation of CD34$^+$ cells. The mean number of colonies obtained in the 2 copies of experiment 2 (Table 2) was used.

the hormone-receptor interfaces, which contributes to the binding energy.[31] In contrast, receptor-receptor interaction in the EPObp$_2$EPO complex seems to be poor, if there is any.[11]

We have demonstrated for the first time that EPO exhibits self-antagonism (Fig 4). The concentration of EPO needed was more than four orders of magnitude higher than that necessary for a maximal response. The likely reason that this effect has not been reported previously is that the micromolar concentrations necessary have not been tested in proliferation assays. The IC_{50} for EPO self-antagonism is approximately 10-fold higher than the IC_{50} for human GH self-antagonism, whereas EC_{50} values for agonism of EPO and GH are similar.[32] There are several explanations possible. The IC_{50} of EPO self-antagonism could be higher due to a lower affinity of site 1 for EPO than for GH. Alternatively, different receptor densities and site 2 affinities could account for the observed discrepancies. Unfortunately, the available data are limited. A well-derived K_d is available for GH (K_d = 0.3 nmol/L),[33] but the corresponding site of EPO has not been as well characterized (K_d, ~0.5 nmol/L).[11] On the other hand, the K_d of site 2 has been well determined for EPO (0.85 to 1.35 μmol/L),[11] but it is unknown for GH. Previous study has demonstrated that there are interactions between the extracellular domains of GH receptor[31]; however, the contributions of the membrane-spanning and intracellular domains to the dimerization of cytokine receptors are poorly understood.

A counterpart to the inactive Fab fragments of MoAb34 would be an EPO mutant that lacks the putative second binding site. Such a mutant would still be able to bind to the receptor, but it would not be able to cause dimerization and therefore should be inactive in a proliferation assay. For GH, Fuh et al[32] have shown that a mutant in which residue Gly-120 is replaced by arginine disrupts the site 2 receptor binding site. This mutant binds to GH receptor with the same affinity as the wild-type hormone, but it cannot stimulate the receptor and it acts as an antagonist. Such an EPO mutant has been described recently[34] and gives further evidence that EPO acts through homodimerization of its receptor.

Why are agonist antibodies for EPO-R so rare? All MoAbs specific to the extracellular domain should dimerize the receptor because they are bivalent. However, the vast majority of antibodies in our screen are not agonists (47 of 48) and form inactive 2:1 complexes. Although generation of specific MoAbs with antagonist activity has been reported previously,[34-37] agonist antibodies have not been described. Apparently, the cell surface imposes steric constraints and the two receptor subunits in the 2:1 complex have to be in a specific orientation and/or distance relative to each other. If this is not the case, receptor-receptor interactions necessary for signal transduction cannot be formed. Stimulation of EPO induces binding of a JAK2 kinase molecule to the cytosolic domain of each EPO-R molecule and increases phosphorylation of EPO-R and JAK2 kinase itself.[38] If the latter is an intermolecular phosphorylation process as has been described for receptor tyrosine kinases,[39,40] close proximity of the two receptor molecules would be essential. Because of the size of the antibody, this proximity is unlikely in most antibody-receptor complexes. Since MoAb34 is a less potent agonist than EPO, it suggests that it dimerizes EPO-R in a slightly different way than the natural hormone does, and that this dimerization is suboptimal for signal transduction.

ACKNOWLEDGMENT

We thank Dr H. Lodish for providing the BaF3 cell line, Dr J. Winkelmann for human EPO-R plasmid pLAP-37, Dr N. Komatsu for the cell line UT-7/EPO, Dr T. Papayannopoulou for OCIM1 cells, and B. van Dyke for the antibody 2E12. We are grateful to Drs H. Lodish and D. Milligan for stimulating discussion.

REFERENCES

1. Krantz SB: Erythropoietin. Blood 77:419, 1991
2. Koury MJ, Bondurant MC: Erythropoietin retards DNA break-